Under "on site" conditions, direct inoculation into cold untempered media of contaminated samples of sea water, followed by incubation 5 to 6 hours later, is a satisfactory technique for routine bacterial analyses.

Evaluating Bacterial Contamination in Sea Water Samples

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IT IS APPARENT that interpretation of the significance of bacterial analyses obtained during surveys of sewage pollution of sea water must be tempered with the realization that many factors influence their reliability. Not the least of these factors is the effect of bacterial die-away or regrowth during the period between sample collection and laboratory analysis.

Standard procedures for the bacteriological analysis of water samples usually recommend that inoculation be carried out as soon after collection as practical and that if storage is necessary the temperature should be kept between the limits of 0° C. and 10° C. Unfortunately, because many sites under survey are remote from adequate laboratory facilities, storage during transportation becomes essential. Needless to say, refrigeration, though desirable, is not always convenient or practical. In the routine practice of public health and pollution control agencies charged with the bacteriological analysis of contaminated waters, delays of 5 to 6 hours are not uncommon. Often storage of samples overnight may be necessary (even though not the preferred procedure) in some overworked laboratories.

Delays or failure to provide refrigeration may significantly influence the results of individual analyses and, consequently, invalidate certain conclusions which might be drawn from the aggregate of results from a certain water under survey. If the water being investigated is that of an ocean bathing beach, the results of analysis may be close to worthless.

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Extent of Coliform Die-Away

The population of the coliform group of bacteria, the common presumptive indicator of domestic sewage contamination, is frequently subject to drastic change during the early hours of its exposure to a new and foreign environment. A review of available literature on the subject of survival of enteric bacteria in sea

water (*1*, *2*), reveals a few sources of information as to the extent of early die-away or growth in sea water samples.

For example, the experiments of Beard and Meadowcroft (*3*) indicate *Escherichia coli* mortalities in the waters of San Francisco Bay of about 68 percent in 0.8 days and 90 percent in 3.5 days.

Ketchum and his associates (*4*), and later Vaccaro and his co-workers (*5*), showed reductions of *E. coli* inoculations as great as 90 percent in raw Vineyard Sound water stored for 24 hours in laboratory containers at room temperature.

Williams (*6*) likewise noted considerable reduction in *E. coli* inoculums in natural sea water stored in the laboratory. He reported an average mortality of 90 percent in 25 hours. In samples of sewage suspended in dialysing tubing in the natural environment the coliform group of organisms suffered a 90 percent mortality in 25 hours although in a few cases growth was experienced.

Nusbaum and Garver (*7*) observed high mortalities of coliforms in laboratory tests, as great as 65 percent in 24 hours, but generally they noted growth rather than die-away in dialysing tubing suspended in San Diego Bay. Coliform survival curves representative of the observations of several of these investigators are shown in the figure.

Several experiments of mine on samples taken from Elliott Bay, Wash., near a large outfall discharging untreated sewage, showed mortalities up to 74 percent in 8 hours of storage. In experiments with a water sample taken from Budd Inlet, a salt water branch of Puget Sound which forms the harbor for Olympia, Wash., coliform die-away was 56 percent in 10 hours at a storage temperature of 20° C. A similar sample held for the same period at 3° C. showed a 38 percent reduction in coliform population. Samples prepared from dilutions of settled sewage and Pacific Ocean water collected off San Francisco showed mortalities as great as 92 percent in 24 hours at 30° C. and 68 percent at 21° C., with no significant change at 5° C.

The presence of organic nutrients may tend to offset bacterial die-away, but without refrigeration growth may occur in the early hours of storage. For example, the addition of lactose broth to a series of samples stored at 20° C. resulted in large increases in population during the first 24 to 48 hours of storage. A period of rapid die-away followed, but die-away occurred only after a substantial lag period. The magnitude of the initial rise, the length of the lag period, and the time for 90 percent mortality were all directly proportional to the concentration of nutrient added. The survival curve for one of these tests (120 p.p.m. lactose broth) also is shown in the figure along with the results of other experiments.

Die-Away Formulation

The figure illustrates certain similarities among the coliform survival curves obtained by different investigators. For example, each curve shows a characteristic logarithmic decrease phase with a slightly varying slope. Several curves, especially those in which growth occurred in the early stages of exposure, show a lag period before the onset of logarithmic decrease. And those experiments for which the data are sufficiently extensive indicate a resistant phase exemplified by a decreasing rate of decrease and the survival of a few coliforms for comparatively long periods of time. The decrease in bacterial numbers after an initial lag is perhaps best expressed by Chick's law (*8*, *9*), which may be written in the following form:

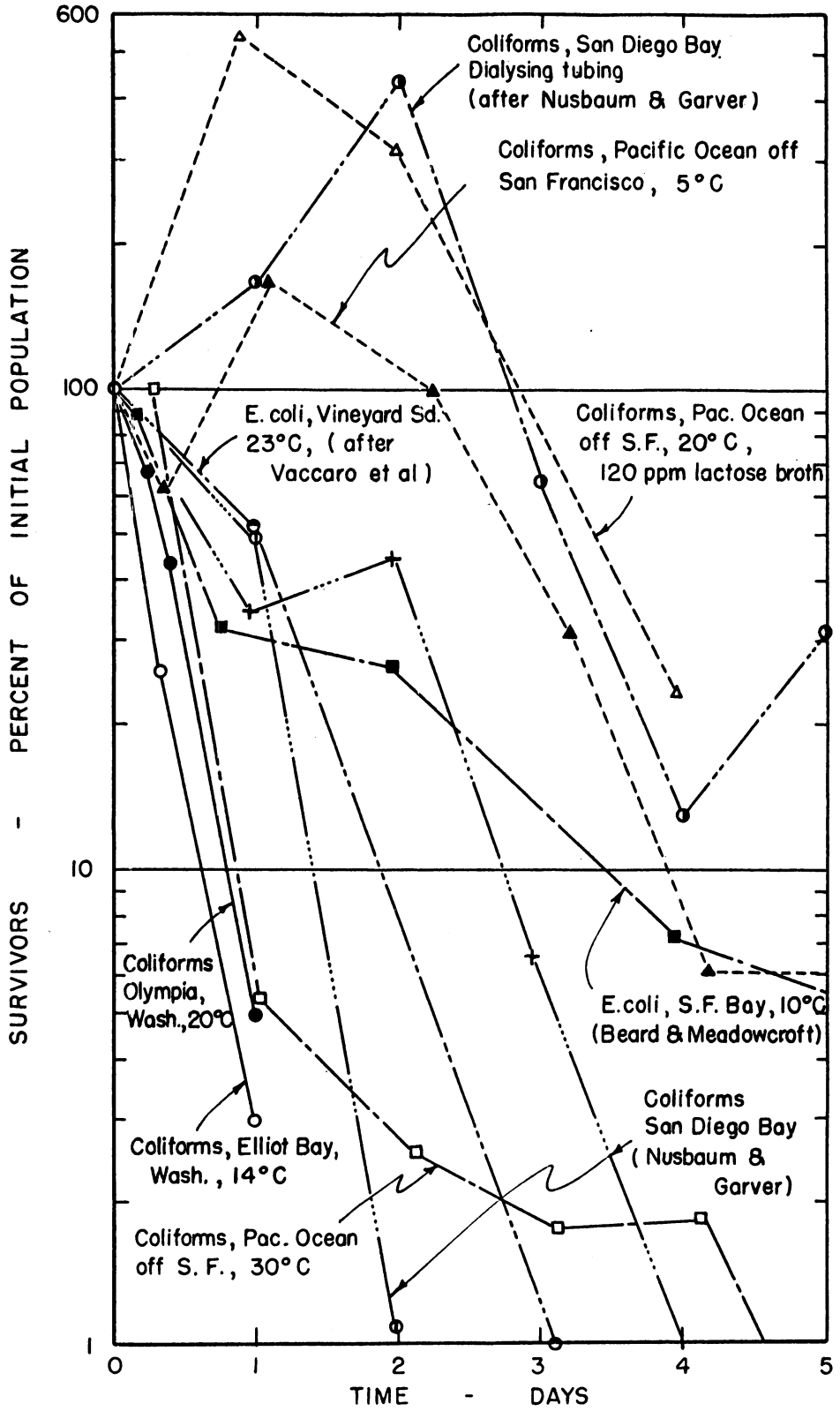
$$\frac{N}{N_o}=10^{-k(t-t_o)}.$$

N is the number of survivors after time t, in days; N_o is the initial bacterial population; t_o is the lag period before logarithmic decrease, and k is a constant.

Field Inoculation Technique

The State of Washington Pollution Control Commission, with due concern for the validity of bacteriological survey results on ocean and brackish waters, has adopted the practice of field inoculation at the site of sample collection in an effort to minimize bacterial die-away or multiplication during transport to the laboratory. This procedure, of necessity, requires that the inoculation be made directly into cold un-

Coliform Survival in Sea Water Samples



tempered media and that the inoculated tubes suffer the delay between sample collection and incubation normally experienced by the raw sample. This practice, while not representing the ideal condition for the preservation of the original bacteria in a viable state, does provide a better chance for an analysis truly representative of "on site" conditions.

The constant k, sometimes referred to as the coefficient of death rate, is indicative of the rate of bacterial die-away. It may be expressed as the reciprocal of the time in days required to achieve a 90 percent mortality of the initial number of bacteria when due account is taken of the initial lag period. It is generally greatest at high temperatures and during the summer months. Typical values of k range from about 0.3 (Beard and Meadowcroft, see figure) to 1.6 (my experiments at Elliot Bay, see figure). The majority of values fall between the limits 0.6 and 1.2.

The significance of the coefficient of death rate in terms of bacterial die-away during sample storage is best illustrated by a simple example. If it is assumed that k has an average value of 1.0 and that there is no appreciable lag, the number of coliforms remaining after 6 and 24 hours' exposure would be, according to the equation, respectively 56.3 percent and 10 percent of the original population.

If a lag period occurs, the sample usually experiences some growth rather than a decrease in population. In either case the bacteriological analysis, unless performed immediately at the site of sample collection, will yield unsatisfactory results. The obvious advantages of refrigeration, where it can be conveniently employed to suppress both bacterial growth and bactericidal activity, cannot be depreciated. It must be acknowledged, however, that under conditions of routine sample collection adequate refrigeration is not readily available. Moreover, when the bactericidal effect of sea water is strong, refrigeration may be somewhat less than satisfactory.

To determine whether or not a preliminary period of 5 to 6 hours in cold media would have any appreciable effect on the viability of bacteria, we compared the cold media inoculation procedure statistically with the tempered media inoculation procedure representative of the best possible practice.

First, from a single sample of settled sewage diluted 1:2,500 in tap water, we prepared twenty 1:10 dilutions in sterile water. Then, we divided the 20 samples into 2 groups of 10 each in order of initial inoculation, placing the odd numbers in group A and the even numbers in group B.

Working alternately from one group to the other, we inoculated all 20 samples according to the standard inoculation procedure and completed the test by confirmation on brilliant green bile broth. All inoculations consisted of five 1-milliliter portions in each of at least 3-decimal dilutions.

Table 1. Comparison of tempered media and cold media inoculation techniques

Sample[1]	Most probable number coliform organisms per 100 ml.	
	Group A[2]	Group B[3]
1	3, 300	1, 700
2	7, 900	2, 300
3	3, 300	3, 300
4	2, 300	4, 900
5	7, 900	4, 900
6	3, 300	4, 600
7	2, 300	2, 300
8	2, 200	2, 300
9	1, 700	3, 300
10	2, 200	4, 900
Arithmetic mean	3, 640	3, 450
Standard deviation	1, 910	1, 170

[1] 1:2,500 dilution of settled sewage in tap water.
[2] Inoculated immediately into tempered media (37° C.) and incubated.
[3] Inoculated immediately into cold media (23° C.). Stored 6 hours at 23° C., then incubated.

We analyzed group A samples in standard strength lactose broth tempered to 37° C. before inoculation, and immediately after inoculation placed the samples in the incubator.

We examined group B samples in the same media but at a temperature of 23° C., and then stored the samples for 6 hours at the same temperature before incubating them at 37° C.

The results of this experiment (table 1) revealed no significant difference between the two procedures. In fact, the spread in individual

observations indicated by the standard deviation from the mean was actually less, though not statistically significant, in group B (cold untempered media) than in group A (tempered media).

To illustrate the effect of short-term changes in bacteria populations in sea water samples and to test the applicability of several storage and handling techniques, we conducted a special series of experiments during an actual bacteriological survey of Budd Inlet, Wash.

We made 14 examinations of the coliform content of the water near a single survey station off the port of Olympia, Wash., from September 1951 through May 1952.

The samples were collected under the wide variety of conditions that would be encountered on any routine field survey. The harbor at Budd Inlet received the untreated sewage discharge from the city of Olympia throughout the 9-month survey period. In addition, the inlet provided the egress for runoff of an extensive area drained by the Deschutes River. A combination of runoff, waste discharge, tidal fluctuation, and climatic variations provided considerable variety in salinity, temperature, amount of dissolved oxygen, and organic content. A summary of individual survey observations is presented in table 2.

We examined each of the samples collected at the survey station by four distinctly different procedures. Immediately after collection of the sample, an inoculation was made into tempered (37° C.) lactose broth as well as into cold media. The temperature of the cold media was close to that of the surrounding air (13° C. to 23° C.). The tempered media series (series A) was incubated without further delay at 37° C. and served as a control for evaluation of other techniques. The samples in the cold media series (series B) were held at air temperature for periods ranging between 5 and 6 hours, a time corresponding to the maximum delay between collection of field samples and inoculation procedures in the laboratory. The samples were then incubated at 37° C.

We divided the original sample into two portions. One (series C) we stored at normal air temperature for 5 to 6 hours, and the other (series D) we refrigerated at 6° C. for a similar

Table 2. Survey observations for State of Washington Pollution Control Commission sampling station, Budd Inlet, Olympia, Wash., 1951–52

Sample	Date	Range of water storage temperature (degrees centigrade)		Storage time (hours)	Dissolved oxygen (p.p.m.)	Biochemical oxygen demand [1] (p.p.m.)	Salinity (p.p.m. NaCl)	Most probable number of coliform organisms per 100 ml.			
		Low	High					A [2]	B [3]	C [4]	D [5]
1	9–14–51	19. 8	21. 4	4. 8	9. 7	3. 0	24, 300	3, 500	2, 400	490	2, 200
2	9–26–51	15. 0	23. 0	5. 6	9. 8	5. 5	28, 500	300	490	490	1, 700
3	10– 4–51	14. 7	19. 8	4. 8	7. 1	2. 2	25, 000	1, 300	2, 400	790	490
4	10–10–51	14. 5	19. 2	5. 0	6. 7	2. 9	26, 000	5, 400	2, 400	1, 700	2, 200
5	10–19–51	13. 0	19. 0	5. 0	6. 4	1. 1	24, 500	3, 500	3, 500	1, 300	490
6	10–30–51	10. 9	20. 3	5. 0	6. 1	1. 4	21, 800	1, 300	1, 400	1, 300	2, 400
7	12–12–51	7. 7	13. 0	6. 0	9. 2	1. 9	11, 100	950	790	330	330
8	1– 9–52	6. 9	21. 4	5. 0	8. 9	2. 6	26, 100	490	490	330	330
9	1–21–52	6. 6	14. 0	5. 8	8. 7	. 7	25, 400	330	490	130	230
10	2– 4–52	7. 4	23. 3	5. 2	9. 1	2. 7	21, 900	3, 500	3, 500	1, 100	460
11	2–27–52	7. 1	17. 7	5. 2	9. 2	1. 1	12, 500	1, 100	790	1, 300	1, 700
12	3–18–52	7. 9	16. 3	5. 0	9. 5	1. 5	24, 900	230	230	330	220
13	4–29–52	11. 0	17. 0	5. 1	10. 7	3. 0	28, 200	330	330	330	330
14	5–29–52	14. 0	17. 0	5. 3	10. 0	3. 1	26, 400	490	490	490	460

[1] Dissolved oxygen consumed during 5 days' incubation of water sample in the dark at 20° C.
[2] Series A (control series). Inoculation into tempered media (37° C.) immediately after collection.
[3] Series B. Inoculation into cold media (storage temperature) immediately after collection; stored 5–6 hours before incubation at 37° C.
[4] Series C. Stored 5–6 hours at indicated temperatures before inoculation into tempered media, then incubated at 37° C.
[5] Series D. Refrigerated 5–6 hours at 6° C. before inoculation into tempered media, then incubated at 37°C.

Table 3. Effect of method of storage and technique of analysis on 14 samples in each series from the bacteriological survey of the State of Washington Pollution Control Commission at Budd Inlet, Olympia, Wash., 1951–52

Procedure [1] for storage and analysis of sample	Most probable number of coliform organisms per 100 ml.				Percent of samples with MPN greater than 1,000	Probability that difference from control is due to chance alone [2] (percent)
	Range	Median	Arithmetic mean	Geometric mean		
Series A (control)[3]	230–5, 400	1, 025	1, 625	990	50	
Series B [3]	230–3, 500	790	1, 407	970	43	[4] 40
Series C [3]	130–1, 700	490	744	592	36	[5] 3
Series D [3]	220–2, 400	475	960	647	36	[6] 17

[1] One analysis in each series from the same original specimen.
[2] Null hypothesis. See reference 10.
[3] Same series identified in text and in table 2.
[4] No significant difference.
[5] Significantly different.
[6] Significant difference questionable.

period. We inoculated each sample after storage into tempered media, and then incubated it in the customary fashion. We used 5 portions of 3-decimal dilutions from each sample to determine the most probable number (MPN) of coliform organisms per 100 ml. of the original sample. A summary of the results of these tests is given in table 3.

These experiments indicate that bacterial die-away during storage, even for comparatively short periods, may seriously affect the interpretation of survey results. It is particularly significant that, even though one series (series D) of samples was refrigerated, an appreciable reduction in bacterial numbers occurred in this series. As we expected, the greatest population change occurred in the portion of the original sample that was stored at normal air temperature before inoculation. This observation is in accord with the results of die-away studies performed on Pacific Ocean water collected near San Francisco in which death rates were observed to be directly proportional to temperature, and refrigeration was noted to induce an appreciable lag prior to die-away (*2*).

Application of the "null hypothesis" (*10*) for testing the significance of the differences in mean values given in table 3 indicates a comparatively small probability that the differences between the mean of the control series A and the mean of either series C or series D could have resulted from chance alone. However, when this statistical test is used to compare series B, the cold media inoculations, with the control series (series A), the difference is not statistically significant. It is readily apparent that when the bactericidal effect of sea water is high a comparatively short delay in inoculation into media, even cold media, may produce a much different picture of contamination than that actually existing at the time of sampling. Even refrigeration of the samples may not be sufficient to arrest bactericidal action although this practice is undoubtedly much superior to storage of the sample at normal air temperatures.

Summary and Conclusions

Changes in populations of coliform bacteria in contaminated sea water during the early hours immediately following collection of the sample may have a significant bearing on the interpretation of the results of bacteriological surveys. Refrigeration of samples, although generally recommended when extended storage of samples is necessary, is not always capable of minimizing the bactericidal effect of sea water. Direct inoculation of the sample into cold media at the sampling site, with a 5- to 6-hour delay in incubation, produces results which are generally comparable with direct inoculation into tempered media followed by immediate incubation. This method is used by the State of Washington Pollution Control Commission and is recommended for general use on bacteriological surveys of sea water.

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